

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 9/127	A1	(11) International Publication Number: WO 96/37194 (43) International Publication Date: 28 November 1996 (28.11.96)
(21) International Application Number: PCT/US96/07303 (22) International Filing Date: 21 May 1996 (21.05.96) (30) Priority Data: 450,142 26 May 1995 (26.05.95) US (71) Applicant: SOMATIX THERAPY CORPORATION [US/US]; Suite 100, 950 Marina Village Parkway, Alameda, CA 94501 (US). (72) Inventors: SULLIVAN, Sean, M.; 133 Rassani Drive, Danville, CA 94506 (US). HOFLAND, Hans; 731 Gonzales Drive, San Francisco, CA 94132 (US). (74) Agents: HALLUIN, Albert, P. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DELIVERY VEHICLES COMPRISING STABLE LIPID/NUCLEIC ACID COMPLEXES (57) Abstract Stable polynucleotide delivery vehicles (SPDV) are described which incorporate a polynucleotide/cationic lipid complex as structural components of the SPDV. The subject SPDVs may optionally incorporate synthetic biodegradable amphipathic lipids, and suitable targeting agents.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

DELIVERY VEHICLES COMPRISING STABLE
LIPID/NUCLEIC ACID COMPLEXES

Related Application

5 This Application is a continuation-in-part of United States Application Serial No. 08/450,142, filed May 26, 1995.

1.0. INTRODUCTION

 The present invention is in the field of biochemistry.
10 In particular, novel compositions are reported which efficiently deliver polynucleotides or other bioactive materials to cells.

2.0. BACKGROUND

15 The present invention relates to novel polynucleotide delivery vehicles, and novel methods for producing the same.

 As the field of molecular biology has matured, a wide variety of methods and techniques have evolved which allow researchers to engineer polynucleotides. Polynucleotides are
20 typically engineered with the goal that they perform a specific function within the cell. Unfortunately, polynucleotide polymers are highly charged molecules (due to the phosphate backbone) and do not readily permeate the cell membrane. As such, concomitant with the advances made in
25 genetic engineering, advances have also been made in methods by which researchers may introduce genetically engineered material into cells.

 One of the methods developed for delivering genetically engineered polynucleotides to cells involves the use of
30 liposomes. The phospholipid bilayer of the liposome is typically made of materials similar to the components of the cell membrane. Thus, polynucleotides associated with liposomes (either externally or internally) may be delivered to the cell when the liposomal envelope fuses with the cell
35 membrane. More typically, the liposome will be endocytosed into the cell. After internalization, the internal pH of the endocytic vesicle may drop substantially, and/or the vesicle

may fuse with other intracellular vesicles, including lysosomes. During or subsequent to the process of vesicle fusion, the internal contents of the endosome may be released into the cell.

- 5 Liposomes are limited as polynucleotide delivery vehicles by their relatively small internal volume of the liposome. Thus, it is difficult to effectively entrap a large concentration of polynucleotide within a liposomal formulation.
- 10 Researchers have tried to compensate for the above inefficiency by adding or using positively charged amphipathic lipid moieties to the liposomal formulations. In principle, the positively charged groups of the amphipathic lipids ion-pair with the negatively charged polynucleotides
- 15 and increase the extent of association between the polynucleotides and the lipidic particles which presumably promotes binding of the nucleic acid to the cell membrane. For example, several cationic lipid products are currently available which are useful for the introduction of nucleic
- 20 acid into the cell. Particularly of interest are, LIPOFECTIN[™] (DOTMA) which consists of a monocationic choline head group which is attached to diacylglycerol (see generally, U.S. Patent No. 5,208,036 to Epstein et al.); TRANSFECTAM[™] (DOGS) a synthetic cationic lipid with lipospermine head groups
- 25 (Promega, Madison, Wisconsin); DMRIE and DMRIE•HP (Vical, La Jolla, CA); DOTAP[™] (Boehringer Mannheim (Indianapolis, Indiana), and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland).

- Properly employed, the above compounds apparently
- 30 enhance the permeability of nucleic acids to cells cultured *in vitro*. Accordingly, the process of lipofection has become an important tool of cellular biology. Typically, formulations comprising the cationic lipids are intermixed with the polynucleotide to be delivered and then applied to
- 35 the target cells. The cationic lipid-polynucleotide complex must generally be used relatively soon after mixing because after a few hours, lipofection efficiency degrades markedly.

From this observation, one may surmise that, at least with respect to lipofection efficiency, the cationic lipid-polynucleotide complex is rather unstable.

From a research perspective, the above complexes are rather facile to prepare. Thus, the relatively short active-life of the prepared complex is not an issue where *in vitro* applications are involved. However, where the medical or *in vivo* use of polynucleotide delivery vehicles comprising cationic lipids is contemplated, one may not assume that a given clinician would necessarily be capable of reliably preparing an active formulation, and subsequently using that formulation within the rather narrow window of optimum activity. Thus, particularly where clinical use is contemplated, a more stable polynucleotide delivery system would be preferred.

Another draw-back of the presently available compounds is that the respective lipid and cationic components are not joined by a biodegradable chemical linkage. As such, most of the presently available synthetic cationic lipids have proven to be significantly toxic because the target cells cannot metabolize the synthetic lipids.

A given level of cellular toxicity may be detrimental but acceptable where *in vitro* or research use of cationic lipids to deliver polynucleotides is contemplated; however, such toxicity is generally unacceptable where *in vivo* use of cationic lipids is contemplated. Thus, cationic lipids which comprise biocompatible, biodegradable, or metabolizable components would be preferred, if not essential, for the preparation of cationic lipid-polynucleotide delivery vehicles for use *in vivo*. Alternatively, cationic lipid-polynucleotide delivery vehicles of substantially reduced toxicity may be employed.

Finally, the currently available methods for using synthetic cationic lipids to transfect cells all produce lipid/DNA complexes which are rapidly inactivated by relatively low concentrations of serum. Serum sensitivity may be easily circumvented in *in vitro* applications by

conducting the initial portions of the transfection procedure in serum free medium. However, serum sensitivity remains a major obstacle to the wide-spread use of cationic lipid-mediated DNA delivery in vivo.

5

3.0 SUMMARY OF THE INVENTION

The present invention contemplates a novel stable polynucleotide delivering vehicle which retains transfection efficiency for at least 48 hours after formation/synthesis.

10 Accordingly, the present invention also claims methods of making stable polynucleotide delivery vehicles which comprise: contacting the polynucleotide to be delivered with an amphipathic cationic lipid conjugate while in the presence of detergent; and removing the detergent whereby
15 substantially size stable polynucleotide delivery vehicles are formed which are also substantially stable with respect to transfection efficiency.

Another embodiment of the claimed invention is a stable complex produced as described above with the added feature
20 that the nucleic acid is complexed with a cation prior to, or concurrent with, the addition of detergent, and the detergent is removed prior to the removal of the cation.

Another aspect of the present invention is a process for making a stable polynucleotide delivery vehicle comprising
25 the steps of contacting polynucleotide with cationic lipid in the presence of detergent, removing the detergent to complex the polynucleotide to the cationic lipid, and isolating the resulting delivery vehicles.

The isolated delivery vehicles may be resuspended in a
30 lesser volume than the volume in which they were originally formed. The result being the formation of concentrated compositions comprising delivery vehicles. Thus, another aspect of the present invention is a stable polynucleotide delivery vehicle generally comprising a DNA concentration of
35 at least about 0.5 mg per ml, and preferably at least about 1.0 mg per ml.

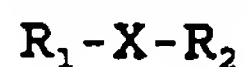
During isolation of the stable polynucleotide delivery vehicles, the toxicity of the resulting composition may be drastically reduced. Thus, yet another embodiment of the present invention is a stable polynucleotide delivery vehicle
5 of substantially reduced toxicity.

The present invention further contemplates stable polynucleotide delivery vehicles which comprise amphipathic cationic lipid conjugates which are additionally complexed with noncationic lipids or other lipid moieties.

10 A further embodiment of the present invention is a stable polynucleotide delivery vehicle which comprises a biocompatible cationic lipid conjugate, and methods for producing and using the same.

As such, the present invention also contemplates
15 biocompatible amphipathic cationic lipid conjugates which comprise a biodegradable lipid moiety which is covalently attached to a biocompatible cationic or polycationic moiety by a pH sensitive chemical linkage which is also biocompatible.

20 Accordingly, an additional embodiment of the subject invention involves biocompatible amphipathic cationic lipid conjugates having the general formula:



wherein R_1 is a biodegradable lipid moiety; R_2 is a
25 biocompatible cationic or polycationic moiety; and X is a biocompatible biodegradable or otherwise labile covalent linker.

Because of the stability of the presently contemplated polynucleotide delivery vehicles, targeting groups may be
30 additionally incorporated into the vehicle whereby the polynucleotides to be delivered may be targeted to particular cell types and/or cellular locales (e.g., the nucleus).

Another embodiment of the present invention contemplates the use of the above stable polynucleotide delivery vehicles
35 to deliver a polynucleotide, or polynucleotides, of interest to a cell. In a related aspect, the stable polynucleotide

delivery vehicles may be used to provide a therapeutic benefit to the individual.

Yet another aspect of the invention is a method of targeting the stable complexes (or delivery vehicles) to particular cells and tissues by associating the complexes with a targeting agent having the property of being capable of binding the stable complex.

4.0. DESCRIPTION OF THE FIGURES

10 Figures 1a and 1b. Show several examples of the novel metabolizable/biodegradable cationic lipids of the present invention. Specifically, examples of N-glutaryl-dioleoylphosphatidylethanolamine conjugated (by a phosphodiester linkage) to hexamine, spermine, spermidine,
15 pentaethylenhexamine (PEHA); N-succinyl-dioleoylphosphatidylethanolamine conjugated to pentaethylenhexamine (by a phosphodiester linkage); 1,2-dioleoyl-sn-glycero-3-succinate (DOSG) conjugated to pentaethylenhexamine (by an ester linkage).

20 Figure 2. Shows an example of the novel biodegradable cationic lipids contemplated by the present invention which incorporate a pH labile linker molecule. A reaction scheme for synthesizing the molecule is also provided.

Figure 3. Shows how and where the cationic groups of
25 some of the presently available cationic lipids are attached to the acyl chains. Specifically, the monocationic synthetic lipids DOTMA, DMRIE, DORIE, and DMRIE•HP; and the polycationic synthetic lipids DOGS (Transfectam™) and DOSPA (Lipofectamine) are shown.

30 Figure 4. Shows the change in the size distribution of transient liposome-DNA complexes at several different time points.

Figure 5. Shows the change in the size distribution of stable polynucleotide delivery vehicles at several different
35 time points.

Figure 6. Shows the relative transfection efficiencies (as measured by beta-galactosidase activity) of transient

liposome-DNA complexes, and stable polynucleotide delivery vehicles as a function of lipid/DNA phosphate ratio and the amount of input DNA used to form the complex/vehicle.

Figure 7. Shows a more discriminating analysis of the relative transfection efficiencies (as measured by beta-galactosidase activity) of transient liposome-DNA complexes, and stable polynucleotide delivery vehicles as a function of the amount of input DNA used to form the complex/vehicle.

Figure 8. Figure 8(a) shows the relative stability (as measured by degradation of transfection efficiency/beta-galactosidase activity) of transient liposome-DNA complexes, and stable polynucleotide delivery vehicles as a function of storage time. Figure 8(b) shows the relative stabilities of the stable and transient complex under different storage conditions. The stable cationic lipid/DNA complexes were stored at minus 20° C with five percent dextrose (solid squares), minus 20° C without dextrose (open squares), 4° C (open circles), room temperature (solid triangles), and 37° C (open triangles). The transient complex was stored at 4° C (solid circles).

Figure 9. Shows the relative stability (as measured by degradation of transfection efficiency/beta-galactosidase activity) of transient liposome-DNA complexes, and stable polynucleotide delivery vehicles as a function of percent serum concentration.

Figure 10. Shows the gene transfer activity of stable cationic lipid/DNA complex after separation from uncomplexed lipid. Stable cationic lipid/DNA complexes were prepared DOSPA/DNA phosphate ratios of 3.3:1, 6.6:1, and 16.5:1. The suspensions were centrifuged and the pellet (solid black box), the supernatant (open box); and the original uncentrifuged suspension (cross-slashed box) were assayed for gene transfer activity. Approximately 0.2 µg DNA from the original suspension was added to 10⁵ NIH 3T3 cells. Corresponding amounts of pellet and supernatant were added to the cells as based upon providing an equivalent volume of the original suspension prior to centrifugation.

Figure 11. Is a graph of the DNA dose used to transfect cells as a function of both β -gal activity and total cell protein.

Figure 12. Shows the comparative levels of expression 5 obtained in targeting studies using an RGD peptide-associated lipid which was incorporated into either transient or stable lipid/DNA complexes at a several different spermine/DNA phosphate ratios, and several different mole percentages of lipid-associated ligands/total lipid.

10 Figure 13. Shows the effect of DOSPA/DNA phosphate ratio on *in vivo* gene transfer, and biodistribution using stable synthetic DNA delivery vehicles that were produced in the presence of cation (MnSV101).

Figure 14. Shows a dose response curve for 15 biodistribution of MnSV101 (as measured by alkaline phosphatase activity) at a DOSPA/DNA nucleotide ratio of 1:1.

Figure 15. Shows a time course for *in vivo* gene transfer and expression by MnSV101 prepared at a DOSPA/DNA nucleotide ratio of 1/1. Alkaline phosphatase expression for 20 each tissue was assayed at designated days by immunocapture.

Figure 16. Shows a comparison between MnSV101 and NaSV101 (formed in the presence of the cation Na instead of Mn) with regards to biodistribution and the efficiency of gene transfer. The DOSPA/DNA nucleotide ratio was kept at 25 1/1, the injection volume was 0.25 ml, and the DNA dose used was 80 ug. Alkaline phosphatase expression for each tissue was assayed by immunocapture. MnSV101 and NaSV101 were either dialyzed against dextrose in the second dialysis step or saline (0.15M NaCl) prior to injection.

30

5.0. DETAILED DESCRIPTION OF THE INVENTION

The biodegradable amphipathic cationic lipids of the present invention may be contacted (ion paired) with a polynucleotide, or polynucleotides, of interest such that the 35 positive charge of the cationic lipid electrostatically interacts with the negatively charged polynucleotide. The electrostatic interaction between the cationic moiety and the

polynucleotide presumably reduces charge repulsion in the polynucleotide and allows the polynucleotide to condense into a more compact configuration (as seen by gel-shift assays).

The condensed cationic lipid/polynucleotide complex subsequently serves as a scaffold or nucleus for the assembly of the polynucleotide delivery vehicle. By physically incorporating the condensed polynucleotide as an integral portion of the structure, the presently described polynucleotide delivery vehicles may stably comprise a more significant proportion of polynucleotide relative to that typically obtained using prior formulations/methods. For example, using the presently disclosed methods, at least about eighty (80) percent of the input polynucleotide remains stably associated with the delivery vehicles of a discrete size-range when measured 48 hours after complex formation.

Preferably, the biodegradable amphipathic cationic lipid conjugates of the present invention comprise biodegradable components. As such, the lipid moiety may comprise any of a number of fatty acids chains (saturated or cis/trans unsaturated) generally having hydrocarbon chains comprising between about 3 to about 26, and preferably between about 12 to about 24 carbon atoms, cholesterol, and derivatives and variations thereof, as long as the lipids are biodegradable or biocompatible.

The cationic component of the present invention may be monovalent, divalent, or preferably polyvalent (i.e., polycationic). The cationic moiety is preferably biocompatible and may comprise any of a variety of chemical groups which retain a positive charge at or near neutral pH including, but not limited to amino groups, amide groups, amidine groups, positively charged amino acids (e.g., lysine, arginine, and histidine), spermine, spermidine, imidazole groups, guanidinium groups, or derivatives thereof.

The cationic component will generally be combined with the polynucleotide at a cation/phosphate ratio that has been optimized for a given application. Usually, the cation/phosphate ratio will be between about 1 and about 20,

often between about 5 and about 17, and preferably between about 6 and about 15. The charge ratio will vary accordingly depending on the number of positively charged groups contained on the cation, and the size of the polynucleotide.

5 Typically, the cationic and lipid components of the claimed biodegradable amphipathic cationic lipid conjugates are described in, or may be obtained from any of a variety of sources including, but not limited to, the 1995 edition of the Merck Index, Budavari, et al., eds., Merck and Company,
10 Inc, Rahway, N.J., the 1995 SIGMA chemical company catalogue, St. Louis, MO., the 1995 Aldrich Biochemicals Catalogue, or the 1995 Ofatlz and Bauer catalogue.

The cationic group may preferably be attached to the lipid component by an ester or phosphodiester bond which
15 renders the fatty acid separable from the cationic group by the action of natural enzymes such lipases or phospholipases, and the like (see Fig. 1). Such a linkage represents an improvement over the currently available synthetic cationic lipids which attach the lipid using an ether bond which
20 presumably contributes to the cellular toxicity associated with the currently available cationic lipids.

For example, Figure 1 depicts the chemical structures for polyamines covalently bonded to dioleoylphosphatidyl-ethanolamine (DOPE) using a glutaryl linker. The DOPE can be
25 degraded by phospholipases A₁, A₂, C, and D. This offers advantages over existing synthetic cationic lipids which use ether bonds for attaching the acyl chains. The ether linkages may not be degraded by phospholipases, and thus the ether linked acyl groups accumulate in the cell membrane.

30 DOSPA, the cationic lipid in Lipofectamine (Life Technology Inc., Gaithersburg, MD) and DOGS, the cationic lipid for transfectam (Promega) contain spermine attached to a diacyl ether-linked glycerol. DOSPA and DOGS are theoretically biodegradable because they contain a peptide
35 bond; however, no corroborating data have been presented in the literature which support this notion. Additionally, even if limited hydrolysis were to occur, the resulting

degradation product would still be an ether linked diacylglycerol.

Another advantage of the presently disclosed biodegradable amphipathic lipids is the way in which the polyamine is attached to the lipid. The diacyl ether linked glycerol for DOSPA and DOGS are attached to the middle of the spermine. The new molecules are attached at the end of the molecule via an amide bond. Figure 3 shows the general formulations for many of the monocation and tetracation lipids which are presently available.

In a particularly preferred embodiment, the cationic and lipid moieties of the claimed biodegradable amphipathic cationic lipid conjugates are covalently linked by a labile (e.g., biodegradable or pH labile) linker group. Labile linkers allow for the production of polynucleotide delivery vehicles comprising cationic lipids which dissociate the lipid and cation moieties after cellular internalization and/or endosomal fusion.

The lipid analog may be engineered such that the lipid product can destabilize or disrupt the endosomal membrane to facilitate the release of the cation/nucleotide complex into the cytoplasm. The lipid hydrolysis product may be a diacylglycerol, lys-phosphoryl or phosphatidyl ethanolamine, monoacylglycerol, triglyceride, or the like.

One embodiment of the present invention is a pH labile linker molecule. This linkage is based on 2-methylmaleic anhydride which forms an acid labile link upon reaction with amino groups. As such, pH labile bonds modified as described above serve as working exemplifications of the claimed pH sensitive/labile covalent linker moieties (which may also include ester linkages).

For the purposes of the present invention, the term amphipathic shall refer to a molecule or compound which comprises at least one substantially polar (i.e., freely miscible in aqueous solvent) region and at least one substantially nonpolar (i.e., freely miscible in organic solvent) region. The term biodegradable cationic lipid shall